THE 1.4-β-GLUCAN CELLOBIOHYDROLASES OF *TRICHODERMA REESEI* QM 9414

A new type of cellulolytic synergism

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1. Introduction

The main cellulolytic activities (glucanohydrolase and cellobiohydrolase) of *Trichoderma reesei* QM 9414 can be efficiently separated by chromatography on DEAE–Sepharose. By isoelectric focusing it can be shown further that both the glucanohydrolase and the cellobiohydrolase activities are composed of several isocomponents, which are immunologically identical. The chromatogram further reveals activity towards Avicel, which is not associated with cellobiohydrolase I (CBH I) and which is immunologically unrelated to this enzyme [1].

Here we compare the amino acid compositions and N-terminal amino acid sequences of the two cellobiohydrolases and report a new type of synergism.

2. Methods

2.1. *Purification of the enzymes*

The purification of CBH I has previously been described [2]. CBH II was obtained in homogeneous form by preparative isoelectric focusing at pH 4.0–6.0 of the material in fractions 105–120 of the DEAE-chromatography as in [1]. The electrofocusing was done essentially as in [3].

2.2. *Enzyme assays*

The enzymic activities against Avicel (cellobiohydrolase activity) and carboxymethyl cellulose (endoglucanase activity) were assayed as in [2].

2.3. *Analytical isoelectric focusing*

Polyacrylamide gel plates (Ampholine PAG plates, LKB-Prod., Bromma) containing carrier ampholytes at pH 3.5–9.5 were used as described by the manufacturer.

2.4. *Amino acid analysis*

Amino acid analysis was performed in a Durrum D-500 analyser after hydrolysis for 24 and 72 h in 6 M HCl at 110°C. Cysteine and methionine were determined on the analyser as cysteic acid and methionine sulphone, respectively, after performic acid oxidation. Serine and threonine were calculated by linear (threonine) or first-order (serine) extrapolation to zero time of the values observed after 24 and 72 h hydrolysis.

2.5. *Amino acid sequence determination*

Prior to the Edman degradation the N-terminal blockage by a pyroglutamyl residue present in both enzymes was removed by treatment with pyroglutamyl aminopeptidase of the reduced and *S*-carboxymethylated enzymes as in [4]. Automatic Edman degradation was performed in a Beckman Sequencer, model 890 C. PTH-amino acids were identified using a Waters HPLC-system.

2.6. *Cellulose degradation experiments*

The activities of the enzymes against crystalline cellulose (Avicel) and filter paper (Whatman no. 1) was measured for the enzymes alone and in combination. Reducing sugars produced were measured as in the standard assay. The conditions used are given in the figure legends.

3. Results and discussion

As shown in [1] the activity towards crystalline cellulose (Avicel) present in culture filtrates of the fungus *T. reesei* QM 9414 can be separated into two immunologically unrelated components by ion-exchange chromatography on DEAE–Sepharose. The properties of the more strongly adsorbed component (CBH I) is well documented; it is a 1.4-β-glucan cellobiohydrolase [5] which is present as several isocomponents, the dominating component having a pI of 3.95 [1,2]. The other component (CBH II) active against crystalline cellulose could be resolved into two isocomponents by preparative isoelectric focusing. The pI values of these components were ~5.0 and ~5.6, respectively (fig.1). The data in table 1 indicates that the amino acid composition of the two enzymes differ, e.g., in the case of alanin.

The two enzymes were found to be resistant to Edman degradation, as both have a pyroglutamic acid residue at the N-terminus of the polypeptide chain. After enzymic removal of this blockage the sequence of the first 20 amino acid residues of the two enzymes could be established (fig.4). (The main glucanohydrolase component also has a pyroglutamic acid residue at the N-terminus [unpublished]). No obvious sequence homology was found within the first 20 residues.

Table 1
Amino acid composition of CBH I and CBH II (mol %)

Amino acid	CBH I	CBH II
Aspartic acid	11.6	12.1
Threonine	11.5	8.0
Serine	11.1	10.9
Glutamic acid	9.0	6.6
Proline	5.5	7.2
Glycine	12.6	9.5
Alanine	6.4	12.8
Half-cystine	4.7	2.8
Valine	4.7	5.9
Methionine	1.4	0.8
Isoleucine	2.4	3.5
Leucine	5.4	7.4
Tyrosine	5.3	4.6
Phenylalanine	3.1	2.3
Histidine	1.0	1.0
Lysine	2.5	2.1
Tryptophan[a]	n.d.	n.d.
Arginine	1.7	2.5

[a] Tryptophan was not determined

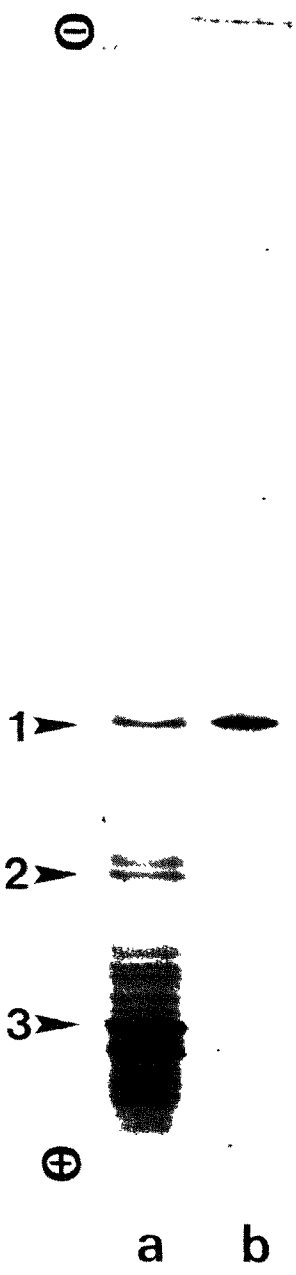

Fig.1. Analytical isoelectric focusing over pH 3.5–9.5. (a) Crude filtrate (a gift from Dr M. Mandels). *T reesei* QM 9414 was grown in a 10 l fermentor on 6% cotton cellulose at 28°C under pH control (pH > 3.0) using the salts medium in [10]. (b) Purified CBH II, pI 5.6 component. Arrows 1 and 2 indicate the position of CBH II pI 5.6 and pI 5.0 components, respectively. Arrow 3 indicates the position of the main CBH I component.

By HPLC the degradation products of the two enzymes when acting on crystalline cellulose was found to be essentially the same, i.e., mainly cellobiose, but also significant amounts of glucose. The activity towards carboxymethyl cellulose, which is usually used to measure endoglucanase activity, was found to be negligible. This justifies the classification, at least tentatively, of the new enzyme as a cellobiohydrolase.

The typical synergism shown by CBH I when acting together with the glucanohydrolases of *T. reesei* is also found for CBH II. This endo–exo synergism has been discussed widely [6–8]. Totally unexpected was the finding that the two cellobiohydrolases show a strong synergistic action (fig.2). This type of synergism has to our knowledge not been described, and a simple explanation, as in the case of endo–exo synergism, is difficult. If the function of the two enzymes simply is to split off cellobiose units from the cellulose chains, the synergism might reflect some type of structural 'asymmetry' in the substrate (e.g., in the hydrogen-bonding pattern of the cellobiose units). Another possibility could be the formation of a complex between the two enzymes, altering the mode of action of the individual components. We have, however, not found any evidence for such an interaction between the two components in free solution, but the possibility that complex formation might be induced by the binding to the surface of the cellulose cannot be excluded. The synergistic action of the two enzymes is also demonstrated by the increased free fibre forming activity (fig.3).

Whether CBH II has been studied in other laboratories is not clear from the literature because a rather detailed study with, e.g., immunochemical techniques is required before the enzyme can be distinguished

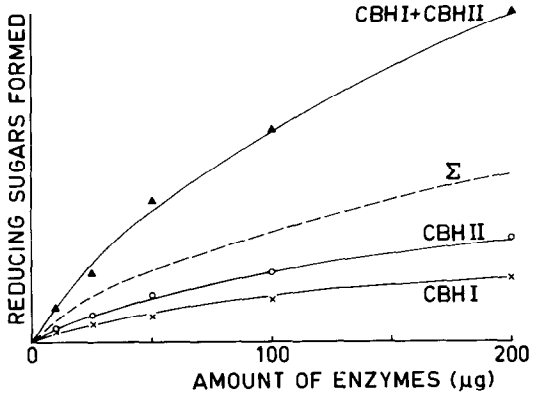

Fig.2. Synergism towards crystalline cellulose. CBH I and CBH II were incubated alone and in combination with 1% Avicel as substrate. The samples were incubated for 1 h at 40°C in 50 mM sodium acetate buffer (pH 5.0). The non-synergistic sum of the activities of CBH I and CBH II is indicated by Σ.

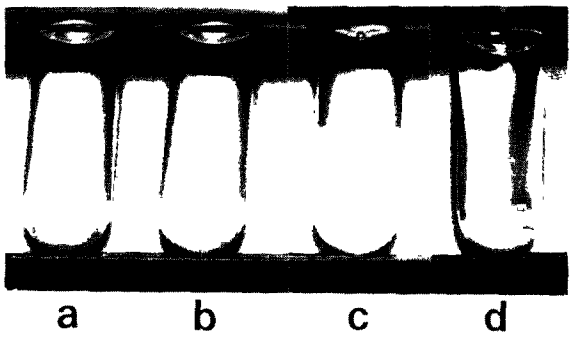

Fig.3. Free fibre forming activity. The two enzymes, CBH I and CBH II, were incubated alone and in combination for 18 h at 40°C in 5 ml 50 mM sodium acetate buffer (pH 5.0) with 50 mg of Whatman no. 1 paper discs as substrate. (a) CBH I (1500 μg); (b) CBH II (300 μg); (c) CBH I (1500 μg) + CBH II (300 μg); (d) control.

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                5                  10                  15                  20
PyGlu - Ser - Ala - Cys - Thr - Leu - Gln - Ser - Glu - Thr - His - Pro - Pro - Leu - Thr - Trp - Gln - Lys - Cys - Ser -

a)

                5                  10                  15                  20
PyGlu - Ala - Cys - Ser - Ser - Val - Trp - Gly - Gln - Cys - Gly - Gly - Gln - Asn - Trp - Ser - Gly - Pro - Thr - Cys -

b)
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Fig.4. The N-terminal amino acid sequence of: (a) CBH I; (b) CBH II.

from CBH I. A highly purified preparation is also needed to demonstrate the most typical property of the enzyme; its synergism with CBH I. Nevertheless, we consider that the enzyme in [9] is identical to the enzyme described here.

From these findings we think it is justified to postulate a new element in the action of the cellulolytic complex, i.e., what we tentatively call exo–exo synergism. A detailed study on the enzymic and physicochemical properties will be published elsewhere.

Acknowledgements

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